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2.	Patent application number *(The Patent Office will fill in this part)*	22 JAN 1998 **9801408.7**
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	UNILEVER PLC UNILEVER HOUSE, BLACKFRIARS LONDON, EC4P 4BQ
	Patents ADP number *(if you know it)*	628003
	If the applicant is a corporate body, give the country/state of its incorporation	UNITED KINGDOM
4.	Title of the invention	FROZEN FOOD PRODUCT
5.	Name of your agent *(if you have one)*	KIRSCH, Susan Edith
	"Address for Service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	PATENT DIVISION, UNILEVER PLC COLWORTH HOUSE, SHARNBROOK BEDFORD, MK44 1LQ
	Patents ADP number *(if you know it)*	5041052002

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7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application		Date of filing *(day/month/year)*
8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.* *See note (d))*	YES		

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11. I/We request the grant of a patent on the basis of this application.

Signature(s) Date: **22 January 1998**

Sandra EDWARDS, Authorised Signatory

12. Name and daytime telephone number of person to contact in the United Kingdom — **Petra Kimber 01234 222893**

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Frozen Food product

Technical Field of the Invention

The invention relates to anti-freeze proteins (AFPs) and frozen food product containing AFPs.

Background to the Invention

Anti-freeze proteins (AFPs) have been suggested for improving the freezing tolerance of foodstuffs.

For the purpose of the invention, the term AFP has the meaning as well-known in the art, namely those proteins which exhibit the activity of inhibit the growth of ice crystals. See for example US 5,118,792.

WO 90/13571 discloses antifreeze peptides produced chemically or by recombinant DNA techniques. The AFPs can suitably be used in food-products. Example 3B shows modified ice crystal shapes if a water-ice mixture is frozen into a film in combination with 0.01 wt% of AFP.

WO 92/22581 discloses AFPs from plants which can be used for controlling ice crystal shape in ice-cream. This document also describes a process for extracting a polypeptide composition from extracellular spaces of plants by infiltrating leaves with an extraction medium without rupturing the plants.

WO 94/03617 discloses the production of AFPs from yeast and their possible use in ice-cream. WO 96/11586 describes fish AFPs produced by microbes.

Several literature places also mention the isolation and/or use of plant proteins for cryoprotection. Cryoprotective proteins have a function in the protection of plant membranes against frost damage. These proteins, however, do not possess recrystallisation inhibition properties and are, therefore, not embraced within the terms AFPs.

Hincha in Journal of Plant Physiology, 1992, 140, 236-240 describes the isolation of cryoprotective proteins from cabbage. Volger in Biochimica et Biophysica Acta, 412 (1975), 335-349 describes the isolation of cryoprotective leaf proteins from spinach. Boothe in Plant Physiol (1995), 108: 759-803 describes the isolation of proteins from Brassica napus. Again, these proteins are believed to be cryoprotective proteins rather than AFPs. Neven in Plant Molecular Biology 21: 291-305, 1993 describes the DNA characterisation of a spinach cryoprotective protein. Salzman in Abstracts and Reviews of the 18th Annual Meeting of the ASEV/Eastern Section in Am. J. Enol. Vitic., Vol. 44, No. 4, 1993 describes the presence of boiling-stable polypeptides in buds of Vitis. Although the proteins are analogous to fish antifreeze peptides, they are cryoprotective proteins and not AFPs. Lin in Biochemical and Biophysical Research Communication, Vol. 183, No. 3, 1992, pages 1103-1108 and in Lin, Plant Physiology (1992) 99, 519-525 describes the 15 kDa cryoprotective polypeptide from Arabidopsis Hakaira. Houde in The Plant Journal (1995) 8(4), 583-593 mentions cryoprotective proteins from wheat.

Up till now, however the use of AFPs has not been applied to commercially available food products. One reason for this are the high costs and complicated process for obtaining AFPs. Another reason is that the AFPs which until now have been suggested for use in frozen food products cannot be incorporated in the standard formulation mix, because they tend to destabilise during processing especially during the pasteurisation step. This destabilisation is believed to be caused by the denaturation of the AFPs; this is a well-known effect commonly observed for peptides and proteins.

In our non pre-published patent application: PCT/EP97/03634 it has been described that AFPs can be isolated from natural sources such as cold-acclimatised grass by means of a new relatively simple process. This process leads for the first time to the identification of AFPs which can conveniently be incorporated in a mix for the preparation of frozen products before the pasteurisation thereof.

This process for the recovery of AFPs from grass involves the steps of

a) isolating a AFP containing juice from the grass;
b) heat treating the grass or the AFP containing juice to a temperature of at least 60°C;
c) removing the insoluble fraction.

Step c of the above process will usually take place after steps a and b. Step a and b can be done in any desired order, for example step a followed by step b (in that case the AFP rich juice will be heated) or step b followed by step a (in that case the natural source will be heated) or step a and b simultaneously.

This process has a number of advantages. Firstly by using the process it is no longer necessary to avoid rupturing of the grass such as required in the processes according to WO 92/22581. This immediately significantly increases the commercial applicability of the process, for example as compared to WO 92/22581, because high investment costs for specific processing are no longer necessary. Also by using the high temperatures it seems possible to extract from a large group of peptides present in the grass a very active AFP which is very active w.r.t. ice-recrystallisation inhibition properties. Thirdly, contrary to expectations, the use of high temperatures does not denature all the proteinaceous material, but does only seem to denature some of the proteins, while the remaining grass AFP have an increased temperature stability. This renders it possible to include the isolated AFPs in compositions which need to be subjected to higher temperatures e.g. a pasteurisation step. This is especially surprising, because for example the AFPs from WO 92/22581 appear not stable under heating conditions.

The process as described above includes in step b the heating of the grass or the AFP rich juice to a temperature of more than 60°C. Preferably the temperature is from 60 to 110 °C, most preferably from 80 to 105°C. The heating step can take place after the isolation of the protein rich juice (step a) or before the isolation of the protein rich juice. Any suitable way to heat the juice can be used, for example conventional or microwave heating, heating optionally with an added extraction medium, steaming etc.

If an extraction medium is used, preferably it is used in small volumes to avoid unnecessary dilution of the AFP fraction. Any suitable extraction medium can be used,

although the use of water is especially preferred. If desired, additives may be added to the water prior to using it as an extraction medium. Most preferred, however water substantially free of additives is used.

Applicants have also found that by the above process a very active AFP derived from grass can be derived. For the purpose of the invention the term grass encompasses members of the Graminae family including for example perennial grasses especially from the Lolium family such as Lolium perenne, Parapholis strigosa, Nardus stricta, Catapodium loliaceum and Lolium multiflorum, from the Poa family such as Poa trivialis, and cereal crops such as winter rye, winter wheat and winter barley. Applicants have determined the amino acid sequence of this AFP.

Surprisingly it has been found that very active AFPs which can be derived from grass are characterised by a high level of the amino acids: Serine (S), Threonine (T) and Asparagine (N). In particular applicants have found that preferred AFPs of the invention are characterised in that at least 40% of the amino acids in the protein are selected from S, T and N.

The preferred molecular weight of AFPs of the invention is form 8 to 16 kDa, more preferred 10-14 kDa, whereby this molecular weight refers to the relative molecular weight on SDS-PAGE of the protein in unmodified form, e.g. in de-glycosylated form.

A second aspect of the invention relates to an AFP which can be derived from grass, said AFP having an amino acid sequence from the N-terminus of:

D-E-Q-P-N-T-I-S-G-S-N-N-T-V-R-S-G-S-K-N-V-L-A-G-N-D-N-T-V-

I-S-G-D-N-N-S-V-S-G-S-N-N-T-V-V-S-G-N-D-N-T-V-T-G-S-N-H-V-

V-S-G-T-N-H-I-V-T-D-N-N-N-N-V-S-G-N-D-N-N-V-S-G-S-F-H-T-V-

S-G-G-H-N-T-V-S-G-S-N-N-T-V-S-G-S-N-H-V-V-S-G-S-N-K-V-V-T-

D-A

Also embraced in the scope of our invention are proteins having a sequence which has a high degree of similarity with the above sequence. For the purpose of the invention all RI active proteins having an amino acid sequence of at least 80% overlap with the above sequence are also embraced in the scope of the invention. More preferred is an overlap of at least 90%, most preferred more than 95%, e.g. those amino acid sequences which differ none or only one or two amino acids with the above sequence. Also isoforms of the above protein are embraced within the invention.

Also embraced within the scope of the present invention are modified versions of the above described proteins whereby said modification does not materially affect the ice recrystallisation inhibition properties, such as glycosylated versions thereof.

The AFP rich juice can be separated from the grass by any convenient process for example pressing, filtering, homogenising, extraction etc. Preferably the grass is made into small pieces or into a slurry before the protein rich fraction is collected, for example by filtering. This maceration can be done by any suitable method, for example

in a blender. It will be well within the ability of the skilled person to divide the material into such a form that collection of the protein rich juice can readily take place.

After collecting and heating (in the desired order) the protein fraction the resulting AFP containing sample can then be treated by any convenient process in order to remove the insoluble fraction and retain the AFP rich liquid fraction. The insoluble fraction can be removed e.g. by filtering, precipitation etc. The AFP rich liquid can then advantageously be further processed to concentrate or isolate the AFPs to bring them in a form suitable for further use. Examples of suitable processes are drying to obtain a powder or paste, further concentration to obtain an AFP concentrate, chromatography to separate the AFPs from the extraction medium etc. Again it will be well within the ability of the skilled person to determine the suitable means and conditions for appropriate isolation.

Applicants have also determined the nucleic acid sequence that encodes the above described AFP. Accordingly a second aspect of the invention relates to a nucleic acid sequence capable of encoding for the AFPs of the invention. Preferably said nucleic acid has the sequence of:

GAT GAA CAG CCG AAT ACG ATT TCT GGG AGC AAC AAT ACT GTC AGA
TCC GGG AGC AAA AAT GTT CTT GCT GGG AAT GAC AAC ACC GTC ATA
TCT GGG GAC AAC AAT AGT GTG TCT GGG AGC AAC AAC ACT GTC GTA
AGT GGG AAT GAC AAT ACC GTA ACC GGC AGC AAC CAT GTC GTA TCA
GGG ACA AAC CAT ATC GTT ACA GAC AAC AAC AAT AAC GTA TCC GGG
AAC GAT AAT AAT GTA TCC GGG AGC TTT CAT ACC GTA TCC GGG GGG
CAC AAT ACT GTG TCC GGG AGC AAC AAT ACC GTA TCT GGG AGC AAC
CAC GTT GTA TCT GGA AGC AAC AAA GTC GTG ACA GAC GCT TAA

Also embraced within the scope of the present invention are alleles or other nucleic acid sequences which are capable to encode the above described AFPs, for example those nucleic acid sequences wherein wrt the above sequence one or more codons have been replaced by their synonyms (i.e. codons encoding for the same amino acid).

Vectors containing a nucleic acid sequence capable of encoding the AFP of the invention are also embraced within the scope of the invention.

Based on the above information it is also possible to genetically modify other natural sources such that they produce the advantageous AFP as identified here-above.

Applicants also have found that AFPs of the above sequence have improved ice-recrystallidation inhibition properties. A suitable test for determining the ice recrystallisation inhibition properties is described in the examples and involves the quick freezing to -40°C followed by storage

for one hour at -6°C. Preferably AFPs which are subject to this test after heat-treatment result in an ice crystal particle size which is less than 5 μm larger than the ice crystal size of a sample with the same AFP which was not heat-treated. Preferably the difference is less than 3 μm, most preferred less than 1 μm.

Preferably those AFPs are chosen which have significant ice-recrystallisation inhibition properties. A suitable test for determining the recrystallisation inhibition properties is indicated in the examples. Preferably AFPs in accordance to the invention provide a ice particle size following an ice recrystallisation inhibition assay -as described in the examples- of 15 μM or less, more preferred from 5 to 15 μm.

The AFP of the invention can conveniently be used in food products, preferably in food products which are frozen or intended to be frozen. Especially preferred is the use of AFPs in products which are heated e.g. by pasteurisation or sterilisation prior to freezing. Especially preferred is the use in frozen confectionery products.

Examples of such food products are: frozen confectionery mixes such as ice-cream mixes and water-ice mixes which are intended to be pasteurised prior to freezing. Such mixes are usually stored at ambient temperature. Suitable product forms are for example: a powder mix which is packed for example in a bag or in sachets. Said mix being capable of forming the basis of the frozen food product e.g. after addition of water and optionally other ingredients and -optional- aeration.

Another example of a suitable mix could be a liquid mix (optionally aerated) which, if necessary after addition of further components and optional further aeration can be frozen.

The clear advantage of the above mentioned mixes is that the presence of the AFP ingredient makes that the mixes can be frozen under quiescent conditions, for example in a shop or home freezer without the formation of unacceptable ice crystal shapes and hence with a texture different to products normally obtained via quiescent freezing.

Very conveniently these mixes are packed in closed containers (e.g. cartons, bags, boxes, plastic containers etc). For single portions the pack size will generally be from 10 to 1000 g. For multiple portions pack sizes of up to 500 kg may be suitable. Generally the pack size will be from 10 g to 5000 g.

As indicated above the preferred products wherein the AFPs are used are frozen confectionery product such as ice-cream or water-ice. Preferably the level of AFPs is from 0.00001 to 0.5 wt% based on the final product. If dry-mixes or concentrates are used, the concentration may be higher in order to ensure that the level in the final frozen product is within the above ranges.

For the purpose of the invention the term frozen confectionery product includes milk containing frozen confections such as ice-cream, frozen yoghurt, sherbet, sorbet, ice milk and frozen custard, water-ices, granitas and frozen fruit purees. For some applications the use in fermented food products is less preferred.

Preferably a the level of solids in the frozen confection (e.g. sugar, fat, flavouring etc) is more than 4 wt%, for example more than 30 wt%, more preferred from 40 to 70wt%.

Frozen confectionery products according to the invention can be produced by any method suitable for the production of frozen confectionery. Especially preferably however all the ingredients of the formulation are fully mixed before pasteurisation and before the freezing process starts. The freezing process may advantageously involve a hardening step, for example to a temperature of -30 Celsius or lower.

Example I

Isolation of AFPs by first heating grass, followed by isolating the AFP rich juice and isolation of the AFP.

Mixed grass tissue (Poa Trivialis, Lolium Perenne, Holcus Lanatus, Bromus Sterilis) was cut in January (mean temperature in that month was 3.5 °C ensuring the appropriate cold acclimatization of the plants). The grass tissue was rapidly transported into the laboratory for further handling and washed thoroughly with water to remove dirt.

500 g of grass clippings was placed in a 650 Watt microwave oven and heated at full power for 5 minutes, whereby the temperature was raised to 85 to 100°C. The grass clippings were then cooled to ambient temperature.

Alternatively the grass clippings are mixed with 500 g boiling water and the mixture is re-heated to 100 °C followed by boiling for 10 minutes under stirring and then allowed to cool to 60 °C.

After the heating step the AFP rich juice was separated from the clippings by filtering. The mass was stirred continuously for 5 minutes in the presence of an equal volume of water and then squeezed through 3 layers of muslin.

The supernatant can be freeze dried to remove the water followed by storage. Alternatively the supernatant can be frozen for storage.

Example II

A liquid premix for the preparation of ice-cream was prepared by mixing:

Ingredient	% by weight
Skimmed milk powder	10.00
sucrose	13.00
maltodextrine (MD40)	4.00
Locust bean gum	0.14
butter oil	8.00
monoglyceride (palmitate)	0.30
vanillin	0.01
AFP (of example I*)	0.01 or none(control)
water	balance

*Note: AFP is added as concentrated AFP solution in some of the water, percentage refers to amount of AFP.

The ingredients were mixed at ambient temperature followed by pasteurisation for 60 seconds at 89°C. The mix was aseptically filled into packs of 500 ml, sealed and stored at ambient temperatures.

The mix can be used for the preparation of ice-cream by whipping it with a conventional house-hold mixer to an overrun of about 70 % followed by freezing under quiescent conditions in a house-hold freezer.

After two months storage the composition according to the invention had a markedly better texture than the control sample.

Example III

The ice recrystallisation inhibition properties of the AFPs can determined as follows:

A sample of an AFP containing product was adjusted to a sucrose level of 30 wt% (If the starting level of the sample was more than 30% this was done by dilution, if the starting level was lower sucrose was added to the 30% level).

A 3 µL drop of the sample was placed on a 22 mm coverslip. A 16 mm diameter cover-slip was then placed on top and a 200 g weight was placed on the sample to ensure a uniform slide thickness. The edges of the coverslip were sealed with clear nail varnish.

The slide was placed on a Linkham THM 600 temperature controlled microscope stage. the stage was cooled rapidly (50 °C per minute) to -40°C to produce a large population of small crystals. The stage temperature was then raised rapidly (50°C per minute) to -6 °C and held at this temperature.

The ice-phase was observed at -6 °C using a Leica Aristoplan microscope. Polarised light conditions in conjunction with a lambda plate were used to enhance the contrast of the ice crystals. The state of the ice phase (size of ice crystals) was recorded by 35 mm photomicrography at T=0 and T=1 hour.

Generally this test can be applied to any suitable composition comprising AFP and water. Generally the level of AFP in such a test composition is not very critical and

can for example be from 0.0001 to 0.5 wt%, more preferred 0.0005 to 0.1 wt%, most preferred 0.001 to 0.05 wt%, for example 0.01 wt%

Any suitable composition comprising AFP and water can be used to carry out the test. Generally, however, it will not be necessary to obtain the AFP in purified form. For practical applications normally it would suffice to prepare a liquid extract or juice of natural material, wherein this extract or juice can then be tested.

This method can be applied for example to the AFP containing extracts as obtained in example I, with or without a concentration step.

The recrystallisation inhibition properties of several samples was measured. The AFP juices obtained after extraction and heating in accordance to example I were measured for their recrystallisation properties as above.

Non heat treated grass extract from grass harvested in January was obtained from Silsoe (UK). The extract was centrifuged for 1 hour to remove soil and insoluble debris as follows, Centrifuge:Sorvall RC3C,Rotor:H6000A, Temperature:+5°C, Rotor Speed:5000rpm(7268g).

A sample of the extract was freeze dried to determine its total solids content. This was found to be 11.48 mg/ml. The dried extract was then rehydrated with 30% Sucrose solution to its original total solids concentration. Several solutions were prepared by diluting the extract as necessary with 30% Sucrose solution.

Ice crystal recrystallisation inhibition activity was measured using the assay as described above.

The T=0 and T=1 hour pictures from the recrystallisation inhibition assays had their mean ice crystal sizes measured using the Zeiss TGA 10 analyser. The ice-crystal size (length) was determined by drawing around the perimeter of the crystals. The maximum length for each individual ice crystal of a batch of ice cream was imported into a spreadsheet where analysis of the data set was carried out to find the mean, and standard deviation.

The results obtained are shown in the table below.

Sample	Total Solids (mg/ml)	Ice Crystal Size (μm)		Ice Crystal Growth in 1 hour at -6°C (μm)
		T=0	T=1 hour at -6°C	
Undiluted	11.48	5.2	7.3	2.1
50% Extract	5.74	5.5	7.6	2.1
25% Extract	2.87	6.3	8.9	2.6
12.5% Extract	1.435	6.6	13.1	6.5
6.25% Extract	0.7175	8.1	14.7	6.6
3.125% Extract	0.359	7.4	17.0	9.6
1.5625% Extract	0.179	9.0	20.3	11.3

These results show the variation in final crystal size and the change in ice crystal size over 1 hour at -6°C for the various dilutions of grass extract. It can be seen that the solids level in the grass extract can be varied in a wide range while still good recrystallisation inhibition properties are obtained. Preferably those concentrations are chosen which result in an ice crystal size after 1 hour of 15 micrometer or less.

A similar test was done with grass extract which had been subjected to heat treatment (10 minutes at 100 °C). No significant deterioration of recrystallisation inhibition properties was seen.

Additionally the grass extracts of example I were tested using the same recrystallisation inhibition test. The following results were obtained:

Heat treatment	Crystal Size in µm T=0	T=1
60 °C 1 hour	9.6	11.1
Boil 10 minutes	9.8	11.3

These results show that even after heating the extract of cold acclimatised grass maintained the ability to inhibit ice crystal growth.

Example IV

Seed of perennial rye grass (Lolium perenne) was supplied by Barenbrug (UK) Ltd. The seed was planted in 9 inch pots in Levingtons No.2 compost and after germination, which usually occurred between 4-7 days after planting, the plants were grown on in the glass house for a further 7-10 days until they were approximately 15-20cm high. The plants were then transferred to a cold room for cold acclimation. Acclimation was for 30 days at +4°C with 8-16 hours light/dark.

Cold acclimated leaves were cut and homogenised in 50mM Tris, 10mM EDTA adjusted to the required pH with concentrated HCl. Homogenisation was for 2x 30s in a Waring blender with a 2.5:1 ratio (w/w) of buffer to leaf.

The homogenate was then placed in a boiling water bath for 10 minutes and collected through four layers of muslin. The collected supernatant was further centrifuged at 15,000 xg in the 8 x 50ml rotor of a Sorvall centrifuge.

Ice recrystallisation properties were tested by the method described in example III.

Heat stable extract (10-50ml) was concentrated approximately ten-fold in an Amicon concentrator using a PM10 membrane. 1ml aliquots of concentrate were desalted on a Fast Desalting Column on a FPLC separation system (Pharmacia). The column was run at 1ml min^{-1} in 50mM Tris/Cl, 10mM EDTA, pH8.5. Fraction (1ml) collection was initiated as soon as the monitored O.D. 250nm started to rise. The RI active fractions were pooled, typically in a volume of 3ml.

The RI activity was loaded onto a Mono Q (HR5 x 5) column equilibrated in the same Tris, pH8.5 buffer. The pass was collected as a single fraction and the column was immediately eluted with a linear gradient of 0-0.25M NaCl over 25 minutes at 1 ml min^{-1}. The whole activity was routinely found to remain unbound eluting solely in the pass fraction.

The pass fraction was readjusted, carefully to pH9.5 using 1 microlitre drops of 4M NaOH and loaded onto the Mono Q column which had been re-equilibrated in Tris buffer pH9.5. The pass was collected as a single fraction and then the column was immediately eluted with a 0-0.25M NaCl linear gradient over 25 minutes. The flow rate was 1ml min^{-1} and 1ml fractions were collected for RI assay.

The active peak fractions from the Mono Q column were pooled, concentrated to 50 microlitre on a PM10 Centricon concentrator and loaded onto a Sephadex 75 column on the Smart system. The column was equilibrated in 50mM Tris/Cl pH9.5 buffer and the flow rate was 50 microlitre min^{-1}. Sample collection was delayed for 1.0 microlitre to allow for the void volume of the column and then 50ml fractions were collected. The active fractions were run on SDS PAGE.

Samples were run on 10% Nu Page gels from Novex. The samples and gel buffer were prepared as described in the manufacturers instructions and gels were stained using a Novex silver staining kit.

Protein samples were also separated by SDS PAGE followed by electroblotting onto PVDF membrane based on the method of Matsudaira (24) in 10mM CAPS buffer, pH 11.0, 10% methanol.

The membrane was wetted in methanol and then equilibrated in Caps buffer for 10 minutes. Blotting was for 16 hours at 20 volts constant voltage. After blotting the proteins were visualised by staining the membrane with 0.2% coomassie brilliant blue in 50% methanol, 1% acetic acid for 1 minute. The membrane was then destained with 50% methanol until the proteins bands were clearly visible. The membrane was washed in Milli Q water, air dried and stored at -20 0C.

The N-terminus of the 29 kDa band was sequenced as follows by conventional methods providing the amino acid sequence of: The 29 kDa protein, believed to be the boiling tolerant RI active was immobilised onto PVDF membrane after separation by SDS PAGE and subjected to protein sequencing of the amino-terminus and 14 of the first 17 residues were successfully sequenced. There was no signal detected at residue 10 but as further amino acids were identifiable at later residues it suggests that the gaps are due to a genuine inability to detect an amino acid. The most frequent reason for this are glycosylated residues which are not soluble in the sequencer solvents and so are not washed from the sequencer cartridge after cleavage from the polypeptide backbone. The 27 kDa protein isolated form Poa pratensis was also sequenced at the N-terminus (Table 2) and shown to have a similar sequence suggesting that it is part of the same protein family. This implies that there is a single class of AFPs responsible for the boiling tolerant RI activity in Gramineae.

Table 2

Alignment of the N-terminal sequences of the 29 kDa boiling tolerant RI active protein from Lolium perenne and the 27 kDa protein from Poa pratensis

Lolium	- D E Q P N T I S G X N T V R X G
Poa	- A E T P N T I S G T N N

Example V

A degenerate oligonucleotide primer (Lol 1) was designed and synthesisied from the protein N-terminus sequence (ASP-GLU-GLN-PRO-ASN-THR-ILE), as GAYGARCARCCIAAYACIAT where Y = C+T, R = A+G and I = Inosine.

First strand cDNA was prepared from 5µg of 30 day cold acclimated Lolium perenne leaf RNA using Superscript Reverse Transcriptase (Stratgene) and an oligonucleotide primer OG1 (GAGAGAGGATCCTCGAG (T) 15) according to the manufacturers instructions. 1% of the first strand cDNA was used as a template, together with Lol1 and OG1 primers, in PCR reactions. The reactions were carried out in a thermal cycler using Taq DNA Polymerase (Gibco BRL) for 30 cycles (1minute at 94°C, 1 minute at 55°C and 1 minute at 72°C) after an initial denaturation step of 2 minutes at 94°C.

A PCR product of ~ 600 bp was amplified and subsequently purified from a 1% agarose gel and cloned into the pTAg vector (R&D systems) according to the manufacturers instructions. The cloned PCR product was sequenced on a Perkin Elmer (Applied Biosystems) automated DNA sequencer using T3 and T7 primers. The nucleotide were substantially similar to:

GAT GAA CAG CCG AAT ACG ATT TCT GGG AGC AAC AAT ACT GTC AGA
TCC GGG AGC AAA AAT GTT CTT GCT GGG AAT GAC AAC ACC GTC ATA
TCT GGG GAC AAC AAT AGT GTG TCT GGG AGC AAC AAC ACT GTC GTA
AGT GGG AAT GAC AAT ACC GTA ACC GGC AGC AAC CAT GTC GTA TCA
GGG ACA AAC CAT ATC GTT ACA GAC AAC AAC AAT AAC GTA TCC GGG
AAC GAT AAT AAT GTA TCC GGG AGC TTT CAT ACC GTA TCC GGG GGG

CAC AAT ACT GTG TCC GGG AGC AAC AAT ACC GTA TCT GGG AGC AAC
CAC GTT GTA TCT GGA AGC AAC AAA GTC GTG ACA GAC GCT TAA

and the deduced amino acid sequence was substantially similar to:

D-E-Q-P-N-T-I-S-G-S-N-N-T-V-R-S-G-S-K-N-V-L-A-G-N-D-N-T-V-
I-S-G-D-N-N-S-V-S-G-S-N-N-T-V-V-S-G-N-D-N-T-V-T-G-S-N-H-V-
V-S-G-T-N-H-I-V-T-D-N-N-N-N-V-S-G-N-D-N-N-V-S-G-S-F-H-T-V-
S-G-G-H-N-T-V-S-G-S-N-N-T-V-S-G-S-N-H-V-V-S-G-S-N-K-V-V-T-
D-A

The open reading frame codes for a protein of ~ 12kDa molecular weight, which is considerably less than the apparent molecular weight on SDS PAGE of 29 kDA. The coding sequence is also observed to contain six recognised glycosylation sites (ASN-X-SER/THR) and a substantial number of SER and THR residues which may also be glycosylated. This evidence suggests that the native protein isolated from Lolium is highly glycosylated. However protein that has been enzymically deglycosylated still retains ice recrystallisation inhibition activity suggesting that the glycosylation is not essential for the protein to be active.

Example VI

To prove that the Lolium perenne cDNA described in example A codes for an AFP, expression of the coding region was carried out. A strain of Pichia pastoris, a methylotrophic yeast, was created containing the Lolium AFP cDNA.

The Lolium cDNA was cloned into a pPIC9 vector with an α - factor signal sequence to ensure secretion from the cell and glycosylation. All enzymes were from Boehringer Mannheim and used according to the manufacturers instructons. Construction of expression vectors, transformation and growth of Pichia were all as described in the Invitrogen Pichia Expression Kit (Version B) Manual.

The Lolium cDNA was cloned into the pPIC9 vector as a PCR amplification fragment, with compatible restriction ends for ligation into the pPIC9 vector. This was produced using Lolium cDNA as the template and the primers GTATCTCTCGAGAAAAGAGATGAGCAGCCGAACACGATT and TTAATTCGCGGCCGCCTGTAGGAAAAGTATGGTATATC which introduced a Xho1 restriction site at the 5' end and a Not1 restriction site at the 3' end of the amplification fragment and ensured that the Lolium cDNA was in frame with the secretion signal open reading frame. The reactions were carried out in a thermal cycler using Taq DNA polymerase and Pfu proof reading enzyme (Boehringer Mannheim) for 30 cycles (1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C). All subsequent PCR reactions were carried out under the same conditions but withiut Pfu enzyme. The Xho1/Not1 cDNA fragment was then cloned into the Xho1/Not1 digested pPIC 9 vector and transformed into competent E Coli cells (strain XL1-blue). After transformation, they were plated

onto LB plates with 50µg ml ampicillin and grown at 37°C for 16 hours. Then, 20 ampicillin resistant transformants were picked and analysed for integration of the Lolium cDNA by PCR using the 5' AOX1 and the 3' AOX1 primers that had been synthesised as specified in the Invitrogen Kit Manual.

Plasmid DNA was isolated from four positive transformants by miniprep and linearised by digestion with Sal1 for transformation into Pichia cells that had been grown and washed as described in the Invitrogen Manual. The linearised DNA digests were electroporated into the Pichia cells using a BioRad GenePulser as recommended and after addition of ice cold 1M sorbitol spread on MD plates and grown at 30°C for 24 hours until colonies were visible. 16 transformants were then picked onto fresh MM and MD plates and transformants that grew normally on the MD plates but more slowly on the MM plates were selected. These transformants were then analysed by PCR using the 5'and 3' AOX1 primers and 8 positives were tested for expression.

Initially the colonies were innoculated into BMGY medium and grown at 30°C for 24 hours after which the cells were spun down and transferred to BMMY medium to induce expression and returned to 30°C for a further 24 hours. Two more additions of methanol to 0.5% were made at 24 and 48 hours to maintain induction. The cells were then removed by centrifugation and an aliquot of the medium adjusted to 30% sucrose and assayed for ice recrystallisation inhibition activity. The medium of all the transformants contained significant RI activity whereas medium produced in the same

way from control Pichia without the integrated Lolium cDNA had no activity.

The above example demonstrates that the protein isolated from Lolium perenne and the corresponding cDNA represent an RI active AFP.

Claims

1. Anti-freeze protein characterised in that at least 40% its amino acids are from the group of Serine, Threonine and Asparagine.

2. Anti-freeze protein according to claim 1 having at least 80% overlap with the following amino acid sequence:

D-E-Q-P-N-T-I-S-G-S-N-N-T-V-R-S-G-S-K-N-V-L-A-G-N-D-N-T-V-I-S-G-D-N-N-S-V-S-G-S-N-N-T-V-V-S-G-N-D-N-T-V-T-G-S-N-H-V-V-S-G-T-N-H-I-V-T-D-N-N-N-N-V-S-G-N-D-N-N-V-S-G-S-F-H-T-V-S-G-G-H-N-T-V-S-G-S-N-N-T-V-S-G-S-N-H-V-V-S-G-S-N-K-V-V-T-D-A

as well as modified versions thereof.

3. Anti-freeze protein of claim 2, wherein the overlap is at least 95%.

4. Anti-freeze protein of claim 3, wherein the overlap is 100%.

5. Anti-freeze protein of claim 1, wherein the protein has been modified by glycosylation.

6. Nucleic acid sequence capable of encoding for the anti-freeze protein of claim 1.

7. Nucleic acid sequence of claim 6, having the sequence:

GAT GAA CAG CCG AAT ACG ATT TCT GGG AGC AAC AAT ACT
GTC AGA TCC GGG AGC AAA AAT GTT CTT GCT GGG AAT GAC
AAC ACC GTC ATA TCT GGG GAC AAC AAT AGT GTG TCT GGG
AGC AAC AAC ACT GTC GTA AGT GGG AAT GAC AAT ACC GTA
ACC GGC AGC AAC CAT GTC GTA TCA GGG ACA AAC CAT ATC
GTT ACA GAC AAC AAC AAT AAC GTA TCC GGG AAC GAT AAT
AAT GTA TCC GGG AGC TTT CAT ACC GTA TCC GGG GGG CAC
AAT ACT GTG TCC GGG AGC AAC AAT ACC GTA TCT GGG AGC
AAC CAC GTT GTA TCT GGA AGC AAC AAA GTC GTG ACA GAC
GCT TAA

and alleles thereof.

8. Frozen food product comprising the anti-freeze protein of claim 1.

9. Food product according to claim 8, being a frozen confectionery product.

10. Method of obtaining an AFP according to claim 1, whereby the AFP is produced by a genetically modified organism.

11. Method according to claim 10, wherein the organism is a microorganism or a plant line.

12. Plant, capable of expressing the protein of claim 1 or 2 and having an increased frost tolerance.

Abstract

Anti-freeze protein comprising at least 40% of the amino acids S,T or N and having at least 80% overlap with the following amino acid sequence:

D-E-Q-P-N-T-I-S-G-S-N-N-T-V-R-S-G-S-K-N-V-L-A-G-N-D-N-T-V-I-S-G-D-N-N-S-V-S-G-S-N-N-T-V-V-S-G-N-D-N-T-V-T-G-S-N-H-V-V-S-G-T-N-H-I-V-T-D-N-N-N-N-V-S-G-N-D-N-N-V-S-G-S-F-H-T-V-S-G-G-H-N-T-V-S-G-S-N-N-T-V-S-G-S-N-H-V-V-S-G-S-N-K-V-V-T-D-A

as well as modified versions thereof.